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IMMUNOLOGICAL STUDIES OF THE SPERM AND SEMINAL FLUID IN THE HORSESHOE CRAB *LIMULUS POLYPHEMUS* L. (MEROSTOMATA)

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The major physiological events of fertilization are (1) sperm-egg interactions prior to attachment, (2) interactions after attachment of the gametes is established, (3) activation of the egg, and (4) union of the two pronuclei to form the zygote nucleus (Monroy, 1965). Important aspects of the interactions include the sperm acrosomal reaction, lysins released from the acrosomal vesicle, gamete attachment, and fusion of the gamete plasma membranes.

Since a sperm usually reacts only with an egg of the same species, some mechanism of specificity is involved. The fact that the sperm normally reacts with the egg and not with other cells in the same organism makes the reaction even more specific. The macromolecules which are involved may be an integral part of the plasma membrane or adsorbed to the gamete surface from material in the reproductive tract. The specificity suggests that macromolecules of the sperm and egg interact in a manner comparable to enzyme-substrate or antigen-antibody reactions (Metz, 1967). The initial gamete attachment may involve electrostatic binding with divalent cations possibly participating. Upon closer contact there would be more specificity due to short range forces and steric factors (Perlmann, 1959). The purpose of chemical investigations of fertilization is to understand the nature and sequence of these events on a molecular level (Monroy, 1965).

Ouchterlony immunodiffusion techniques and cross absorption-agglutination experiments with a number of crustacean decapods have indicated that there are at least three surface antigens of *Limulus* sperm (Mowbray, Brown, and Metz, 1970). The present study is a further investigation of *Limulus* sperm antigens.

Materials and Methods

Specimens of the horseshoe crab *Limulus polyphemus* L. were obtained from Florida Marine Biological Specimen Company, Panama City, Florida and Marine Biological Laboratory Supply Department, Woods Hole, Massachusetts. The animals were kept in "Instant Ocean" aquaria with circulating, aerated artificial sea water at 15° C.

Preparation of antigens

Spawning of gametes from male or female animals was artificially induced by electrical stimulation (Shrank, Shoger, Schechtman and Bishop, 1967). For sperm studies semen from several animals was combined to obtain approximately

3 ml. The semen contained about 9×10^9 spermatozoa/ml. Centrifugation at $480 \times g$ at 5° C for 30 min, separated the semen into three layers: (1) sperm cells, (2) seminal particles, and (3) seminal plasma. The seminal particles and seminal plasma were centrifuged again for better separation. The sperm layer was diluted to 10.6 ml with sea water and centrifuged again in order to wash the spermatozoa.

Sonification was used to break the sperm cells into three parts—acrosomal caps, nuclei, and flagella. A 10% sperm suspension was fixed in 2% formaldehyde in sea water in order to prevent cellular disintegration during the sonification process. Another sperm component, the axial rod, was exposed by treating sperm cells with double strength sea water, thus producing the "false acrosome reaction" (Andre, 1963; Shoger and Brown, 1970). Unsuccessful attempts were made to induce the true acrosome reaction.

Soluble sperm antigens were obtained in frozen-thawed extracts made with liquid nitrogen. One ml of concentrated spermatozoa was fixed with 2 ml of Tris buffer (pH 7.4) and was frozen and thawed three times. This treatment formed an extremely viscous mass. Viscosity was reduced by adding two drops of 0.05% deoxyribonuclease (Nutritional Biochemical Corp., 1 crystallized). Frozen-thawed extracts were also made with concentrated seminal particles, diluted with an equal volume of Tris buffer.

In some experiments, frozen-thawed sperm extract was absorbed with whole eggs to remove any sperm antigens which react with the egg surface. One drop of sperm extract was mixed with at least 500 eggs for this absorption. The same results could usually be obtained with about 300 eggs, but with fewer eggs absorption was apparently not complete.

An egg extract was prepared from 1 ml eggs in 4 ml Tris buffer. This suspension was sonified 1 min, frozen-thawed twice at −20° C, and centrifuged at $3020 \times g$ to remove large pieces of cellular debris. A heart extract was also made by crushing, sonifying, and freezing heart tissue (washed extensively) in Tris buffer.

Preparation of antibodies

Antibodies to *Limulus* sperm antigens were produced in rabbits. Spermatozoa mixed with physiological saline and Freund's complete adjuvant (Fisher Scientific Co., Chicago, Illinois) were injected subscapularly. The sera from different rabbits were usually pooled. Both serum and the extracted globulin fraction (150 mg protein/ml) were used from the experiments. The globulin was obtained by adding an equal volume of saturated ammonium sulfate to cold serum. Both serum and globulin were absorbed with whole sperm cells. Absorbed and unabsorbed antibodies were then used in agglutination, immunodiffusion, and immunoelectrophoresis experiments. In all cases in which the globulin fraction and the whole serum were used and compared, the results were similar. Antibodies to *Limulus* eggs and heart tissue were obtained by methods like those described for the sperm.

Agglutination experiments

Agglutination tests (Quinn, 1968) were used to determine the presence of surface antigens on the sperm and seminal particles. One drop of the antiserum or

globulin fraction to be tested was added to one drop of a suspension of spermatozoa or seminal particles. The following materials were used as controls: (1) rabbit normal sera, (2) anti-egg sera, (3) anti-heart sera, (4) anti-sperm sera absorbed with whole sperm cells, and (5) sea water. Each type of test was repeated at least three times with the same results.

Immunodiffusion and immunoelectrophoresis analysis

Ouchterlony immunodiffusion tests (Ouchterlony, 1968; Crowle, 1960) and immunoelectrophoresis (Nerenberg, 1966; Cawley, 1969) were used to study the number and distribution of soluble sperm antigens. The optimum antigen and antibody concentrations for immunodiffusion and immunoelectrophoresis tests were determined by varying the concentrations of both reactants. The results reported in Table II are based on at least ten trials. The minimum number of consistent bands are shown.

Results

Agglutination tests

Agglutination experiments were performed on whole spermatozoa, "false reacted" spermatozoa, separated sperm parts, and seminal particles in order to test for surface antigens (Table I). The agglutination capacity of the following agents was tested: (1) anti-sperm sera, (2) anti-sperm sera absorbed with whole spermatozoa, (3) anti-egg sera, (4) anti-heart sera, and (5) normal sera.

Spermatozoa agglutinated upon addition of whole anti-sperm sera (Figs. 1 and 2). The spermatozoa were attached in the following ways: (1) head to head, (2) flagellum to flagellum, and (3) head to flagellum. The head-to-flagellum agglutination was least prevalent. Spermatozoa which had undergone a "false acrosome reaction" agglutinated in the same manner as unreacted spermatozoa. The axial rod was not involved in the attachment between sperm cells.

Anti-sperm sera absorbed with whole spermatozoa, anti-egg sera, anti-heart sera, and normal sera initially had no noticeable effect on sperm suspension. Head-to-head agglutination of spermatozoa did occur in all four sera after approximately 1 hr. Normal sera diluted 1:80 did not cause agglutination within 6 hrs, whereas anti-sperm sera of the same dilution caused agglutination of spermatozoa within 1 hr.

Sonification broke spermatozoa into nuclei, acrosmal caps, and flagella (Fig. 3). Anti-sperm sera caused agglutination primarily between like parts (Fig. 4). To a lesser extent, caps were seen adhering to flagella (Fig. 5). As in the agglutination tests with whole sperm cells, the other types of sera had no visible effect at first, but in approximately 1 hr nonselective agglutination of acrosomal caps and nuclei began to occur.

The seminal particles are all approximately spherical in shape, but they vary in size and refractiveness (Fig. 1). Seminal particles of all sizes were strongly agglutinated by anti-sperm sera (Table I). Agglutination between particles of different sizes shows that these particles have at least one antigen in common, but not all of the seminal particles are necessarily alike. Anti-sperm sera absorbed with sperm cells produced the same effects on the particles as did whole anti-sperm

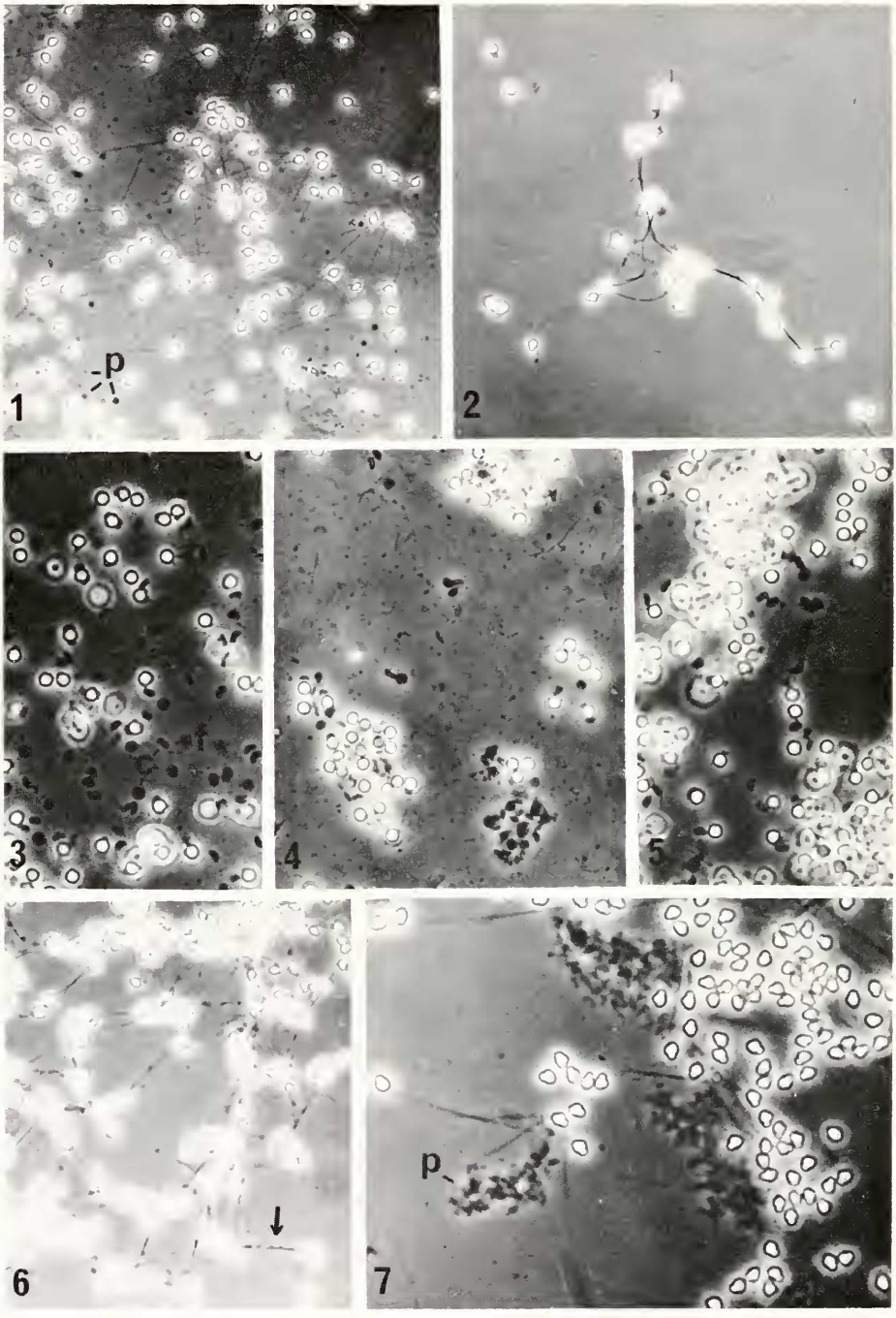

FIGURE 1. Semen diluted 1:10 with sea water. Nonrefractive seminal particles (p) of various sizes can be seen among the spermatozoa, × 250.

sera in agglutination tests. When whole semen was used, some seminal particles became attached to the sperm flagella (Fig. 6). This phenomenon did not occur with control sera. When anti-sperm sera absorbed with sperm cells was used with whole semen, the particles agglutinated but did not attach to sperm flagella (Fig. 7).

These agglutination tests using whole sperm, dissociated sperm parts, and seminal particles demonstrated at least three sperm surface antigens and two surface antigens on the seminal particles. One of the surface antigens is shared by the seminal particles and the sperm flagella. The seminal particles have at

TABLE I

Results of agglutination experiments. Agglutination tests were performed with anti-sperm serum. Symbols indicate degree of agglutination of seminal components (sperm, sperm nucleus, etc.) listed horizontally to those listed vertically

	Sperm	Sperm nucleus	Acrosomal cap	Flagellum	Axial rod	Seminal particle
Sperm	**	—	—	—	0	*
Sperm nucleus	—	**	0	0	—	0
Acrosomal cap	—	0	**	*	—	0
Flagellum	—	0	*	**	—	*
Axial rod	0	—	—	—	0	0
Seminal particle	*	0	0	*	0	**

** Strong agglutination.
* Moderate agglutination.
0 No agglutination.
— No test performed.

least one surface antigen which is not present on the sperm surface since the particles are agglutinated by sera absorbed with whole sperm.

Immunodiffusion and immunoelectrophoresis analysis

Information about the number and location of sperm antigens and their relationships to antigens of the seminal fluid, the egg, and the heart was provided by immunodiffusion and immunoelectrophoresis tests. Frozen-thawed sperm extracts

FIGURE 2. Washed spermatozoa agglutinated by anti-sperm serum. Note head to head, head to flagellum, and flagellum to flagellum attachments; sperm dilution: 1:20, × 250.

FIGURE 3. Washed spermatozoa broken into parts by sonification. Note highly refractive nuclei (n), nonrefractive acrosomal caps (c), and flagella (f). Sperm cells fixed with 2% formaldehyde before sonification; sperm dilution: 1:25, × 400.

FIGURE 4. Sperm parts agglutinated by anti-sperm serum. Note segregation of nuceli and acrosomal caps into separate aggregates, × 400.

FIGURE 5. Sperm parts agglutinated by anti-sperm serum. Note attachment of several acrosmal caps to one flagellum, × 400.

FIGURE 6. Whole semen, diluted 1:10, and anti-sperm serum. Note seminal particles attached to the flagella of the agglutinated spermatozoa, × 250.

FIGURE 7. Whole semen and anti-sperm serum absorbed with whole sperm cells. Note that the spermatozoa are not agglutinated, but agglutination of the seminal particles is considerable. Light pressure applied to the slide would cause the sperm to drift apart but not the particles, × 400.

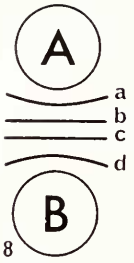

FIGURE 8. Immunodiffusion; (A) anti-sperm serum, (B) frozen-thawed sperm extract from washed spermatozoa; (a), (b), (c), (d) precipitation bands.

produced a minimum of four bands with anti-sperm globulin (Fig. 8; Table II). Immunoelectrophoresis of the frozen-thawed extracts yielded at least seven bands (Fig. 9).

Tests with absorbed anti-sperm sera were made to determine the number of surface and subsurface antigens (Table II). Sperm extracts reacting with absorbed sera produced only two precipitin bands as compared with four to seven bands formed with unabsorbed sera (Figs. 9 and 10a). These experiments revealed that a minimum of five soluble antigens are associated with the sperm surface of *Limulus*. Anti-sperm sera absorbed with "false reacted" sperm cells produced the same result as anti-sera absorbed with normal sperm cells.

Immunodiffusion tests revealed that some of the sperm antigens are related to antigens found in the other seminal components (Table II). There are at least three soluble antigens associated with the seminal particles in *Limulus*. Two of these antigens are identical to two soluble subsurface sperm antigens represented by bands "a" and "b" (Figs. 8 and 10a). Antigen "b" is also found in the seminal plasma. Another antigen which is present in the seminal plasma and seminal particles is apparently somewhat related to sperm subsurface antigen "a" (band "a," Fig. 8).

TABLE II

Results of immunodiffusion experiments. Anti-sperm sperm was used to test for the presence of sperm antigens in the extracts and seminal plasma. Sperm surface antigens were distinguished from subsurface antigens by reacting sperm extract with serum absorbed with whole spermatozoa. Absorption of sperm extract with whole eggs was used to test for the presence of sperm antigens which react with the egg surface. This table summarizes the results shown in Figure 10

Antigens†	Sperm extract	Sperm surface	Particle extract	Seminal plasma	Egg extract	Sperm extract absorbed with eggs	Heart extract
a	*		*		*	*	*
b	*		*	*	*	*	*
c	*	*					
d	*	*					
Related to a††			*	*			

* Antigen present.

† Antigens a, b, c, and d are demonstrated by bands in Fig. 8.

†† The band for this antigen, show in Fig. 10a, fuses with band "a" but is not identical to it.

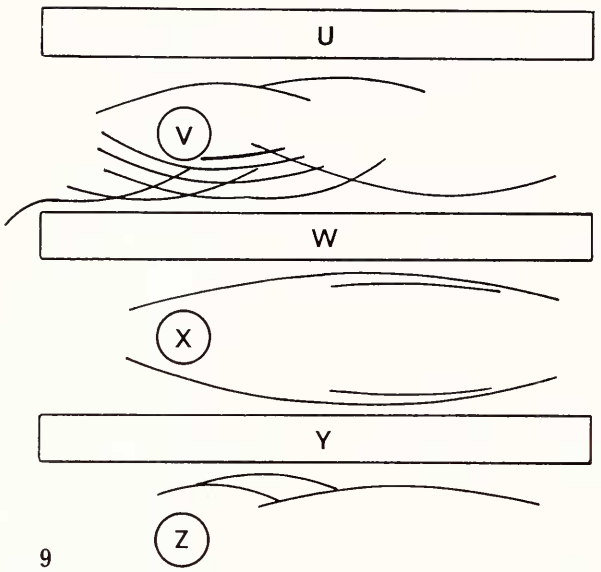

FIGURE 9. Immunoelectrophoresis; (U) anti-sperm serum absorbed with whole sperm cells, (V) frozen-thawed sperm extract from washed spermatozoa, (W) anti-sperm serum, (X) seminal plasma, (Y) anti-sperm serum, (Z) frozen-thawed seminal particles extract.

The sperm cells also have antigens in common with *Limulus* eggs and heart tissue (Figs. 10b and 10c; Table II). Sperm subsurface antigens "a" and "b" are found in both egg and heart extracts.

Frozen-thawed sperm extracts absorbed with whole eggs formed two precipitin bands with anti-sperm sera (Fig. 10b). These two bands correspond to bands "a" and "b" obtained with whole sperm extracts (Table II).

DISCUSSION

Soluble sperm antigens have been studied in a variety of species by means of immunodiffusion techniques. The *Limulus* sperm has at least seven antigens,

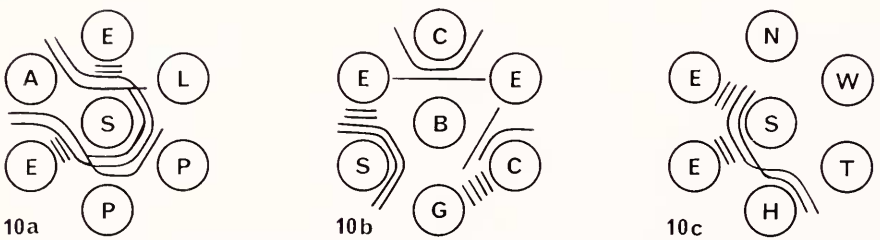

FIGURE 10. Immunodiffusion; (A) anti-sperm serum absorbed with whole sperm cells, (B) frozen-thawed sperm extract absorbed with whole eggs, (C) anti-egg serum, (E) frozen-thawed sperm extract, (G) frozen-thawed egg extract, (H) frozen-thawed heart extract, (L) seminal plasma, (N) normal serum, (P) frozen-thawed seminal particles extract, (S) anti-sperm serum, (T) anti-heart serum, (W) sea water.

whereas four antigens have been identified on the sea urchin sperm (Metz, 1967). Studies on mammals have revealed different numbers of antigens associated with the spermatozoa: (1) four in the guinea pig (Katsh and Katsh, 1961), (2) seven in the bull (Hunter and Hafs, 1964), (3) ten in the Chinese hamster (Piko and Tyler, 1962), and (4) fourteen in the rabbit (Hunter, 1969). The number of sperm antigens demonstrated for *Limulus* is within the range of numbers reported for other organisms in different phyla.

Particular interest is directed toward sperm surface antigens since the initial interactions of the gametes in fertilization must involve macromolecules on the surface of the sperm and egg. Immunoelectrophoresis revealed five sperm surface antigens in the case of *Limulus*, and agglutination tests showed that the antigens are unevenly distributed on the surface. At least one antigen is apparently unique to the surface of the nuclear part of the sperm. The agglutination of acrosmal caps to flagella was probably due to identical or very similar antigens on these sperm parts. Thus the distribution of surface antigens on the *Limulus* sperm may resemble the situation found in the sea urchin sperm (Metz, 1967).

Possible functions which sperm surface components play in fertilization have been summarized by Piko (1969). They are as follows: (1) prevention of sperm agglutination, (2) regulation of membrane permeability, (3) prevention of an early acrosome reaction by covering receptor sites on the plasma membrane, and (4) prevention of an early uptake of the sperm by phagocytes. The antigenic components of the *Limulus* sperm may be involved in similar functions.

Immunological studies have shown that some mammalian spermatozoa become coated with materials from the seminal fluid (Hunter and Hafs, 1964; Weil and Rodenburg, 1962; Hunter, 1969). These findings indicate the importance of studying antigens in the seminal fluid and determining their relationship to the sperm. The present study on *Limulus* indicates that there are two soluble subsurface sperm antigens which are also associated with the seminal particles. One or both of these antigens could be involved in the agglutination of the seminal particles by sera absorbed with whole sperm. The antigen which is found on the surface of the sperm flagellum and on the seminal particles may be insoluble and thus not subject to detection by immunodiffusion tests.

The distribution of antigens in *Limulus* semen is comparable to that in some mammals. For example, some of the antigens of the bovine sperm are also found in the seminal plasma (Hunter and Hafs, 1964). The same situation exists in the Chinese hamster (Piko and Tyler, 1962). Seminal particles possessing some antigens in common with the sperm have been found in the bull (Lindahl and Brattsand, 1962) and the rabbit (Metz, Hinsch, and Anika, 1968). The latter studies showed that the rabbit seminal particles have antigens which are also present in the seminal plasma, and at least one particle antigen is shared by the sperm surface. These results resemble those obtained for the *Limulus* system.

Ouchterlony experiments involving cross reactions between egg or sperm extracts and anti-sera show antigenic relationships between the gametes (Table II). The results suggest that the sperm and egg have two antigens in common. Since these antigens are subsurface ones, at least in the case of the sperm, they are probably not directly involved in the initial sperm-egg interactions. The same two antigens are also found in extracts of *Limulus* heart tissue, so they may be

associated with sperm cell structures which do not participate directly in fertilization. A comparative study should be pursued to determine whether these antigens are specific for *Limulus*.

One comparative study dealing with the sperm surface antigens of five crustacean decapods and *Limulus* has been performed (Mowbray, Brown, and Metz, 1970). Cross-absorption-agglutination tests were used to study the species specificity of the sperm surface antigens. This work revealed that at least two sperm surface antigens are not entirely unique to *Limulus*. The *Limulus* sperm shares one surface antigen with the sperm of *Pagurus pollicaris* and another surface antigen with the sperm of *Cancer irroratus*. Since fertilization reactions are usually very specific ones, unique *Limulus* sperm antigens should be distinguished from those which are also found in other types of *Limulus* cells or other species.

The experiments using sperm extracts absorbed with whole eggs suggest that the two surface antigens represented by bands "c" and "d" on Ouchterlony plates react with the egg surface (Fig. 10b). A fertilizin-antifertilizin type of system may be present. Further investigations may reveal that these antigens are involved in the acrosome reaction and initial gamete attachment.

Further immunological studies using isolated sperm parts can be performed for more specific localization of sperm antigens. Such studies combined with staining techniques and enzyme analysis can provide information about the chemical nature of the antigens. The purpose is aimed at the eventual elucidation of biochemical reactions involved in fertilization in *Limulus*.

Summary

Immunological methods were used to investigate *Limulus* sperm antigens. Immunoelectrophoresis experiments revealed seven soluble sperm antigens, and absorption techniques demonstrated that five of these antigens are located on the sperm surface.

Agglutination tests showed that surfaces of the sperm nucleus, acrosomal cap, and flagellum have both different and common antigens. The surface of the acrosomal rod is nonantigenic.

Immunodiffusion experiments revealed that the spermatozoa, seminal particles, and seminal plasma have some antigens in common. The seminal particles are strongly agglutinated by anti-sperm sera and moderate agglutination occurs between particles and sperm flagella.

Cross reactions of anti-sperm sera with *Limulus* egg and heart extracts demonstrated that two sperm subsurface antigens are also present in these other types of cells. Absorption techniques combined with immunodiffusion experiments gave evidence that two sperm surface antigens react with components on the egg surface.

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